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DNA-methylase from regenerating rat liver: purification and characterisation

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ABSTRACT

DNA methylase has been purified 660-fold from nuclei from regenerating rat liver. The enzyme is able to methylate single stranded (ss) and double stranded (ds) DNA, the only reaction product being 5-methylcytosine. Previously unmethylated double stranded DNA from prokaryotes (M.luteus) as well as from eukaryotes (Ascaris suis) can serve as substrates. The synthetic copolymers (dG-dC)n • (dC-dG)n and (dG,dC)n are also methylated. While SV40 DNA is almost not methylated, PM2 DNA is a good substrate even in the supercoiled form. The enzyme methylates 1 in 17 bases in heterologous M.luteus DNA, but only 1 in 590 in homologous rat liver DNA. The high methylation level of M.luteus DNA, an analysis of the methylated pyrimidine isostichs and a preliminary dinucleotide analysis suggest that all the CpGs in a DNA can be methylated.

INTRODUCTION

Although it has been known for serveral years that eukaryotic DNA generally is methylated in about 4 % of its cytosine (1,2), the biological function of this phenomenon has not yet been revealed. Only in prokaryotes part of the DNA methylation, adenine - as well as cytosine-methylation, is known to be involved in the modification-restriction mechanism (3), and, moreover, it has been suggested that the only methyl-groups of ϕ x 174 plays a role in the development of the phage (4).

Recently Holliday (5) and Riggs (6) raised the question whether eukaryotic DNA methylation might control cell differentiation. Indeed differences in the level of methylation in different tissues of an organism (7,8) could be taken as a support for their hypothesis. The authors postulate the existence of an enzyme which, during cell division, conserves the stable state of methylation of the differentiated cell by methylating the

newly synthesized strand only opposite to a methyl group in the parental strand. The enzyme, therefore, should not be able to methylate double stranded unmethylated DNA.

To test this assumption and to come to more direct evidence about the role of DNA methylation it seems reasonable to purify and characterize eukaryotic DNA methylase(s). There have been recent reports about DNA methylases from various sources (9,10,11), showing, besides many features in common, some important controversial points. E.g. the sequence specificity of the methylase has not yet been well clarified, nor has the question whether eukaryotic methylases are able to methylate double stranded unmethylated DNA.

We report here the partial purification and characterization of a DNA methylase from regenerating rat liver, an enzyme which has been described to some extent previously (12,13,14).

MATERIALS AND METHODS

Radiochemicals and scintillation media: S-adenosyl-L methyl-^{3}H methionine (SAM) (5 - 10.5 Ci/m mole, 1 m Ci/ml) was obtained from Amersham Buchler (England) and diluted to 2.2 Ci/m mole, 0.2 m Ci/ml with unlabeled S-adenosyl-L-methionine from Boehringer Mannheim (West-Germany). A toluene or a toluene /Triton x 100 (3:1) scintillation medium was used. Toluene scintillation medium contained 0.01 % POPOP and 0.4 % PPO.

Enzymes and Chemicals: RNase A and pronase were from Boehringer Mannheim (West-Germany). RNase was heated for 10 min at 90° C, and pronase was selfdigested for 16 h at 37° C before use. S_1-nuclease (Aspergillus) was purchased from Sigma Chemie München (West-Germany). Hemoglobin was from Serva Heidelberg (West-Germany), bovine serum albumin, yeast alcohol dehydrogenase (ADH) and S-adenosyl-L-homocysteine SAH) from Boehringer Mannheim (West-Germany). Phosphocellulose and DEAE cellulose and DEAE cellulose De 52 were obtained from Whatman (England) and DEAE-Sephadex A50 from Pharmacia Uppsala (Sweden). Seakam agarose, ethidium bromide were from Serva Heidelberg (West-Germany).

DNAs and synthetic polynucleotides: E. coli B DNA was prepared by the Marmur procedure (15), M. luteus DNA was from

Miles (England), salmon sperm and calf thymus DNA from Serva Heidelberg (West-Germany). Ascaris suis larval DNA was a donation of Dr. G. Roth, München. M. luteus DNA was further purified by RNase A and pronase treatment, extracted twice with chloroform-isoamyl alcohol, and precipitated with isopropanol (15).

Supercoiled PM2 phage DNA was isolated by the procedure of Gray et al. (16) after growing the phages as described by Espejo and Canelo (17). SV40 DNA was prepared in the same way as PM2 DNA. PM2 phages were generously donated by Dr. R. Schäfer, Basel. SV40 by Dr. M. Koch, Berlin. DNA from normal rat liver was extracted by the method of Marmur (15) from rat liver nuclei prepared as described below. (dA-dC)n • (dT-dG)n was from Boehringer Mannheim (West-Germany). The synthetic copolymers (dC)n, (dC)n • (dG)n, (dC-dG)n • (dG-dC)n, (dC,dG)n, (dT,dC)n were obtained from P-L Biochemicals Milwaukee (USA).

Enzyme assay: As the optimal salt concentration is not the same for the methylation of native and denatured DNA (12), different incubation mixtures were used. The mixture for denatured DNA, which was routinely used in enzyme purification, contained in a total volume of 0.2 ml, 10 mM Tris HCl buffer, pH 7.8, 5 mM EDTA, 0.1 mM Dithiothreithole, 0.02 % Triton X 100, 150 mM KCl, 4.5 µM SAM (= 2 µCi), 5 µg of denatured DNA (10 min 100°C) and the enzyme.

The assay for methylation of native DNA differed in that, the KCl concentration was 50 mM and in that 5 µg native DNA was used. The other ingredients were the same as for denatured DNA.

After an incubation of 30 min at 37° C the reaction was stopped in ice, 0.5 ml of water, 0.25 ml of 0.2 M sodium pyrophosphate, 250 µg of salmon sperm DNA added, and the DNA precipitated by 2 ml of 15 % TCA. After 10 min in ice the precipitate was collected on GFC filters (Whatmann, England) and washed 8 times with 5 ml of 5 % TCA containing 10 mM sodium-pyrophosphate and twice with ethanol. After drying at 100° C for 10 min the filters were counted with 5 ml of toluene scintillation medium. This procedure was usually used in enzyme purification. If RNA- and protein-methylation had to be strictly excluded we used the method proposed by Kalouzek and Morris (18)

with the exception that after the first NaOH treatment the DNA was TCA-precipitated, collected on filters and washed as described above. There was about 20 % RNA and protein methylation in the nuclear extract, less than 5 % after the DEAE Sephadex chromatography and 0 % after phosphocellulose.

The label was shown to be in 5'-methyl-deoxycytidine by enzymatic hydrolysis of the reaction product and paper chromatography in a borate buffer system (19).

One unit of DNA methylase is defined as the amount of enzyme catalyzing the incorporation of 1 picomole of methyl groups into denatured M. luteus DNA in 1 hour, under standard assay conditions.

Digestion by S_1-nuclease: If incubation of in vitro methylated DNA with S_1-nuclease was intended, EDTA was omitted from the methylation assay. After methylation of the DNA the assay mixture was filled up to 0.4 ml with water, and extracted with one volume of chloroform-isoamyl alcohol and two volumes of ether successively. The mixture was divided into two equal parts one of which was heated at 100° C for 10 min to denature the methylated DNA. Incubation of this part with S_1-nuclease served as a control for the efficiency of the S_1-treatment. Both parts were brought to 0.6 ml containing: 66 mM sodium-acetate, pH 4.6, 1 mM Zn SO_4, 200 mM NaCl, 10 µg denatured salmon sperm DNA and 100 units of S_1-nuclease. After 1 h at 37° C the mixture was treated further as described for the methylation assay.

Preparation of isopyrimidine stichs: M. luteus DNA was long-term methylated in vitro (see legend to fig. 2), carrier DNA added, and the mixture extracted with chloroform-isoamyl alcohol and treated with NaOH, as described above. After TCA precipitation of the DNA and washing once with ethanol/ether 3:1 the isopyrimidine stichs were prepared and separated on DEAE-cellulose as described by Sneider (20).

Electrophoresis of PM2 DNA: PM2 DNA was methylated in the assay for ds DNA as described above. 100 µl of the assay mixture containing 2.5 µg DNA were separated on 1.4 % agarose gels in 0.036 M Tris/HCl, 0.03 M NaH_2PO_4, 1 mM EDTA, pH 7.5, at 7 V

per tube for 2.5 hours (21). After staining with ethidium bromide the bands with the linear, open circular and supercoiled DNA (about 80 % of the DNA) were excised and boiled each in 5 ml 20 mM sodiumpyrophosphate to solubilize the agarose. After addition of 250 µg carrier DNA and cooling, the DNA was TCA-precipitated, washed once with TCA, hydrolyzed in 5 % TCA for 40 min at 90° C, and counted with toluene Triton scintillation medium.

RESULTS

Purification of DNA methylase from rat liver:

Preparation of nuclei: Male Wistar rats (200 g) were killed 25 h after partial (2/3) hepatectomy, their livers excised, and homogenized with 10 volumes of 0.32 M sucrose, 5 mM $MgCl_2$ in a 60 ml potter with 7 strokes at 900 rpm. After centrifugation at 800 g for 15 min the crude nuclear sediment was taken up in 2.7 M sucrose in 5 mM $MgCl_2$ to a final concentration of 2.05 M sucrose and centrifuged in a Beckman rotor 19 at 19 000 rpm for 130 min. 210 g of liver can be treated in one run of this rotor. The nuclei were taken up in 1 M sucrose, 1 mM $MgCl_2$, and sedimented again at 1 000 g for 20 min. These nuclei can be kept frozen at -70° C for a year without loss of activity.

Extraction of the DNA methylase: Nuclei from 60 g rat liver were extracted with 0.8 M KCl in 50 mM Tris buffer, pH 7.8, 0.5 % Triton X 100 for 30 min in a total volume of 9 ml. The KCl concentration was then lowered to 0.3 M by addition of 10 mM Tris, pH 7.8, and after standing for 30 min with occasional stirring the insoluble chromatin was sedimented at 20 000 g for 15 min, the enzyme remaining in the supernatant.

DEAE-Sephadex A50 batch: The nuclear extract was diluted 4-fold with 20 mM Tris, pH 7.8, 1 mM EDTA, 0.5 mM DTE (buffer A), and stirred for 1 h with 22 ml DEAE-Sephadex A50 (column-packed volume) which had been equilibrated with 85 mM KCl in buffer A. After adsorbtion of the enzyme the gel was centrifuged at 2 500 g for 5 min, taken up with 50 ml of 85 mM KCl in buffer A, and sedimented again. The gel was then poured into a column and the enzyme eluted with 0.28 M KCl in buffer A, dis-

carding the first 15 ml and collecting the next 20 ml which contained the enzyme.

DEAE-Sephadex A 50 gradient: The enzyme solution was diluted twice with buffer A, adsorbed to a DEAE-Sephadex A 50 column (1.4 x 5 cm) which had been equilibrated with 0.1 M KCl in buffer A and eluted with 45 ml of a linear gradient from 0.1 M KCl to 0.4 M KCl in buffer A plus 10 % glycerol. The enzyme was eluted as one peak of activity at 0.17 M KCl.

Phospho cellulose step: The pooled peak fractions from the DEAE-Sephadex A 50 column were diluted 2-fold with buffer A containing 0.1 % Triton X 100 and loaded on a phospho cellulose column (diameter 1.2 cm, length 0.7 cm). After washing with 3 column volumes of 0.25 M KCl, 0.1 % Triton, in buffer A, the enzyme was eluted in 1.3 ml 0.55 M KCl, 0.1 % Triton X 100 in buffer A.

Glycerol gradient centrifugation: 0.65 ml of the enzyme solution was layered on a 5 ml 10 - 30 % glycerol gradient in 0.5 M KCl, 0.1 % Triton X 100 in buffer A and centrifuged in a SW 65 Beckman rotor at 50 000 rpm for 14 h. The enzyme banded in about the middle of the gradient under these conditions. A purification scheme is shown in table I.

The enzyme was purified 660-fold over nuclei by this proce-

TABLE I. Purification of rat liver DNA methylase.

Fraction	Protein (ug)	Total Units (p moles CH_3/h)	Specific Activity (units/mg protein)	Purification (fold)	Recovery %
Nuclear crude extract	107 000	7 600	7.1		
Nuclear supernatant	48 500	10 600	20.5	2.9	100
DEAE-Sephadex step	13 700	9 500	69.2	9.6	95
DEAE-Sephadex gradient	1 900	8 600	452.0	64.0	85
Phospho-cell. step	275	5 300	1 930.0	272.0	52
Glycerol gradient centrifugation	62	2 900	4 680.0	660.0	29

Protein was determined by the method of Lowry (21). For details see text.

dure. During the purification the enzyme behaved as one peak of activity on DEAE-Sephadex, on phospho cellulose (if the step elution was substituted by a KCl gradient), as well as on the glycerol gradient. From these procedures we had no indication of multiple DNA methylases. The purified enzyme preparation was stable at -70° C for at least 1/2 year, but still rather unstable at 4° C.

Characterization of the enzyme:

Molecular weight: The approximate molecular weight was determined according to Martin and Ames (22) on a 5 ml 5 - 20 % sucrose gradient in o.5 M KCl in buffer A with ADH, bovine serum albumin, and hemoglobin as standards, centrifuging in a SW 65 Beckman rotor at 42 000 rpm for 13 h (fig. 1). A molecular weight of 115 000 was obtained which is in close agreement with the m.w. of 120 000 reported for the Hela enzyme by Roy and Weissbach (9).

Substrate specificity: The enzyme was assayed with several different natural DNAs and synthetic deoxyribonucleotide poly-

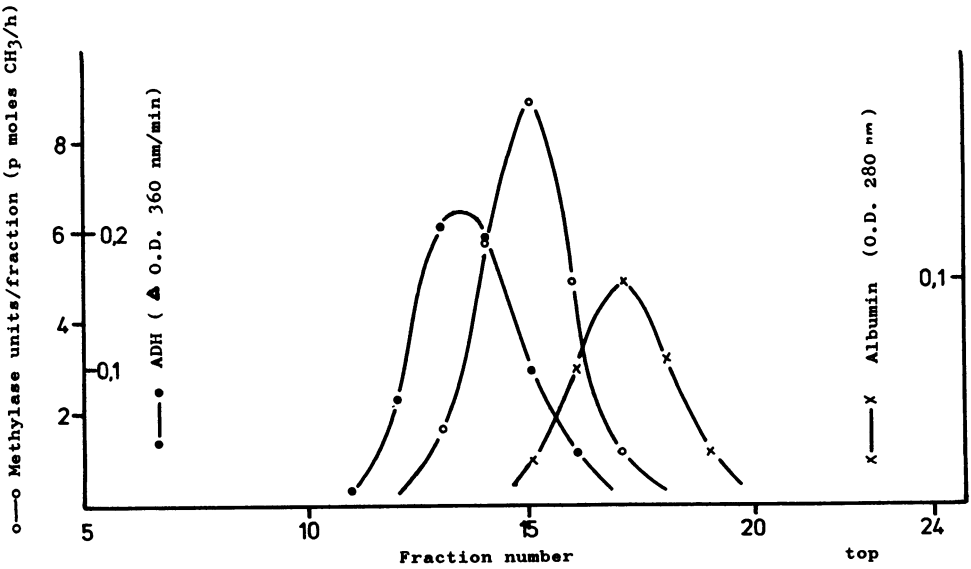

Fig. 1: Sucrose gradient centrifugation of rat liver DNA methylase. 50 units of DNA methylase were centrifuged on a 5 ml 5 - 20 % sucrose gradient with 20 µg ADH, 50 µg bovine serum albumin, and 50 µg hemoglobin (not shown) as marker proteins (see text).

mers which is shown in table II. The purified enzyme seems to prefer single stranded over double stranded DNA.

Turnbull and Adams (11) had recently shown that the methyl groups incorporated into "double stranded" E. coli DNA by a Krebs II ascites cell methylase were in fact in single stranded portions of the DNA. We therefore tested the ds DNAs for S_1-nuclease resistance after methylation (see methods) and could confirm that the counts obtained, when using ds DNA as substrate, were indeed in ds DNA. 95 - 100 % of the cpm in ds DNA were S_1-resistent, whereas only 5 - 10 % of the control were not digested by S_1-nuclease. M. luteus DNA, an unmethylated bacterial DNA (24), turned out to be the best natural substrate in the single stranded as well as in the double stranded form. The DNA of the eukaryote Ascaris suis which does not contain methyl groups either (Dr. R. Baur from our laboratory, unpublished data) is a good ds substrate, too. Of the synthetic copolymers tested (dG-dC)n•(dC-dG)n and (dG,dC)n were relatively good substrates in the double stranded form, whereas (dA-dC)n•• (dT-dG)n, (dT,dC)n and (dC,dC)n were not at all methylated.

While SV40 DNA was not significantly methylated neither in the supercoiled nor in the extended form (after sonication), the DNA of the phage PM2 which is of comparable length and base composition was a good substrate even in the supercoiled form. This was verified by extraction of the PM2 DNA after methylation and separation of the supercoiled form by agarose gel electrophoresis as described in methods.

Kinetic data: With the purified enzyme and in excess of substrates the reaction was linear for at least 40 min and then continued over several hours at a slowly declining rate, depending on the enzyme concentration which evidently affects the stability of the enzyme.

When the extent of methylation for M. luteus DNA was determined with the purified enzyme the reaction finished at a level of 1 methyl group per 17 bases for single stranded DNA and 1 methyl group per 85 bases for double stranded DNA (fig.2). When on the other hand the enzyme fraction after the DEAE-Sephadex gradient was used, saturation of M. luteus DNA was reached at 1 methyl group per about 18 bases for both ss and

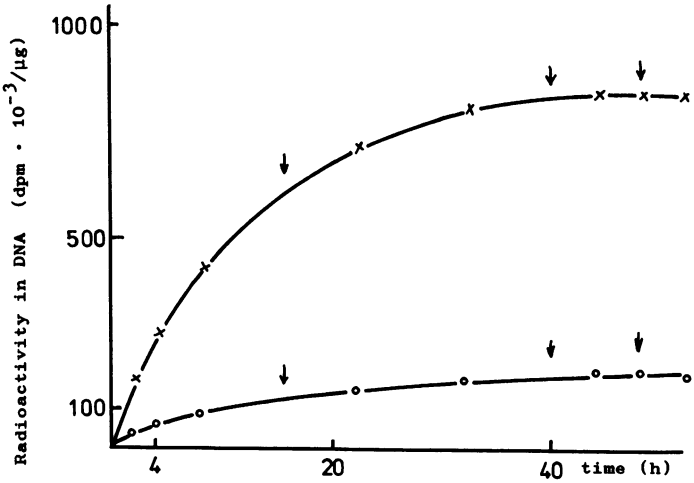

Fig. 2: Long-term methylation of M. luteus DNA.
1 µg of native or denatured M. luteus DNA was incubated with 70 units of DNA methylase in the standard assay for ds or ss DNA. At times indicated by the arrows further 20 units of enzyme and 0.45 n moles (= 1 µCi) SAM were added.
o—o native DNA, x—x denatured DNA.

ds DNA. This indicates a loss of ds activity of the enzyme during the purification (see discussion). The extent of methylation for (C,G)n under ds conditions was 1 methyl group in 19 bases, for (dG-dC)n•(dC-dG)n 1 in 22, for ascaris suis DNA 1 in 85, and for homologous rat liver DNA 1 in 590.

The Km for ss M. luteus DNA, determined in siliconized glass tubes, was 0.5 µg/ml which is 7-fold lower than reported for the Hela enzyme (9). The Km for SAM, in excess of ss M. luteus DNA, was 2.5×10^{-6} M. SAH, the product of the reaction was a potent competitive inhibitor with a Ki of 3.3×10^{-7} M (fig. 3).

Mechanism of methylation: According to the results of Drahovsky and Morris (12) the DNA methylase from rat liver, after a temperature dependent binding step, moves along ds DNA during its catalytic action. As Turnbull and Adams (11) could not confirm this mechanism for the purified methylase from Krebs II ascites cells, we reexamined Drahovsky's hypothesis with our methylase preparation.

The binding of the enzyme to ds DNA is inhibited by NaCl. Whereas, once the binding has taken place in the absence of

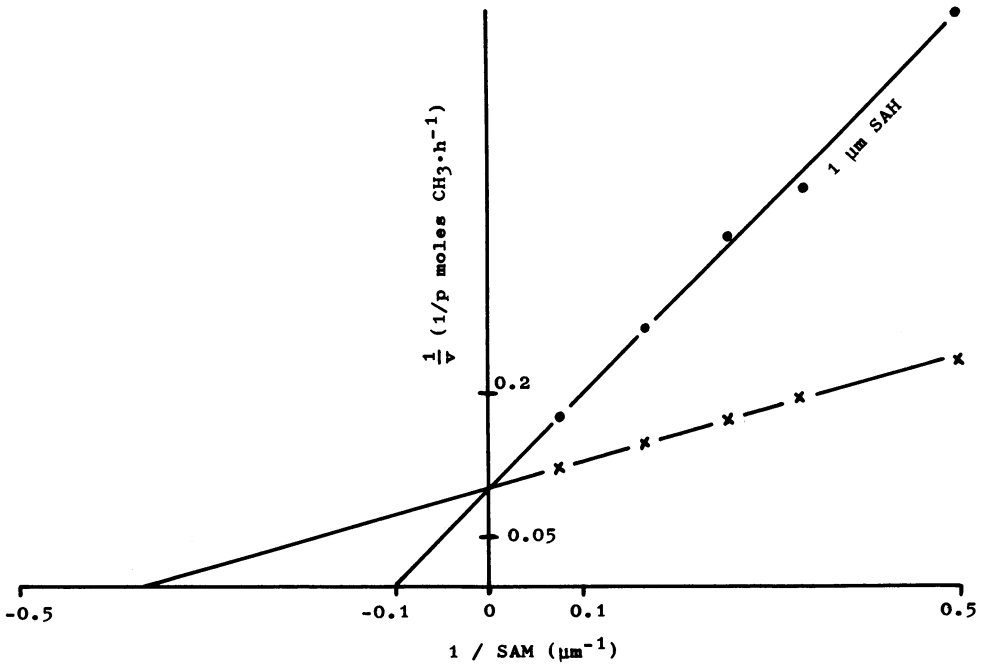

Fig. 3: Double reciprocal plot of DNA-methylase-activity and SAM concentration with and without SAH.

NaCl, methylation can proceed even in the presence of salt (12). We therefore compared the activity of the enzyme on ds DNA at different NaCl concentrations with and without preincubation of enzyme and DNA for 5 min at 37° C in the absence of salt (fig. 4).

Because of its higher activity on ds DNA we used the enzyme after the DEAE-Sephadex gradient for these experiments. The cpm were verified to be in ds DNA by S_1-treatment. The results showing the formation of a salt resistant complex of enzyme and ds DNA on preincubation in low salt are in good agreement with Drahovsky's hypothesis.

Reasons for the different behaviour of the methylase from Krebs cells might be that the enzyme itself or its complex with DNA is more sensitive to salt or that the enzyme is altered by the purification.

Methylation of pyrimidine isostichs: I has not yet been excluded that different methylases exist for the different sequences which have been shown to be methylated in eukaryotic

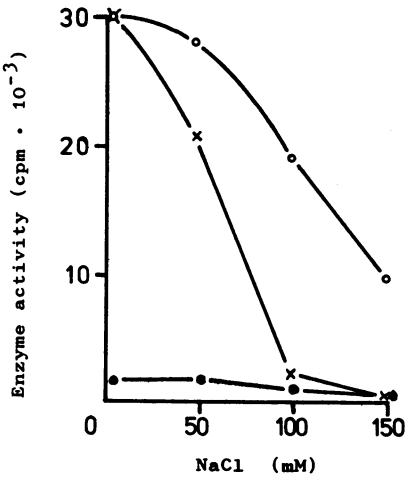

Fig. 4: Effect of salt on methylation of ds M. luteus DNA. DNA methylase was incubated with 5 µg native M. luteus DNA at different NaCl concentrations for 20 min under standard assay conditions, without preincubation x___x or with 5 min preincubation of DNA and enzyme at 37° C in the absence of SAM and salt o___o. After the methylation assay the DNA was incubated with S_1-nuclease as described in methods. Denatured controls from the preincubated assay were equally digested by S_1-nuclease •___•.

DNA (25). By analyzing the isopyrimidine stichs methylated in vitro we tested whether we had purified a methylase specific for only one distinct sequence (fig. 5). Methyl groups were found in all the pyrimidine isostichs separated by the method in a similar distribution as reported for in vivo methylation in hepatoma cells (20). And although this is a rather crude analysis, it shows that the considerably purified enzyme still has a broad sequence specificity.

DISCUSSION

The DNA methylase from regenerating rat liver has been purified 660-fold from nuclei. In contrast to the DNA methylase from Novikoff-cells (10) and Krebs ascites cells (11) showing a certain heterogeneity, the enzyme from rat liver eluted as a single peak of activity on DEAE-Sephadex and phospho cellulose as well as on glycerol gradient centrifugation.

The analysis of the pyrimidine isostichs methylated in vitro by the purified enzyme (fig. 4) shows a similar pattern

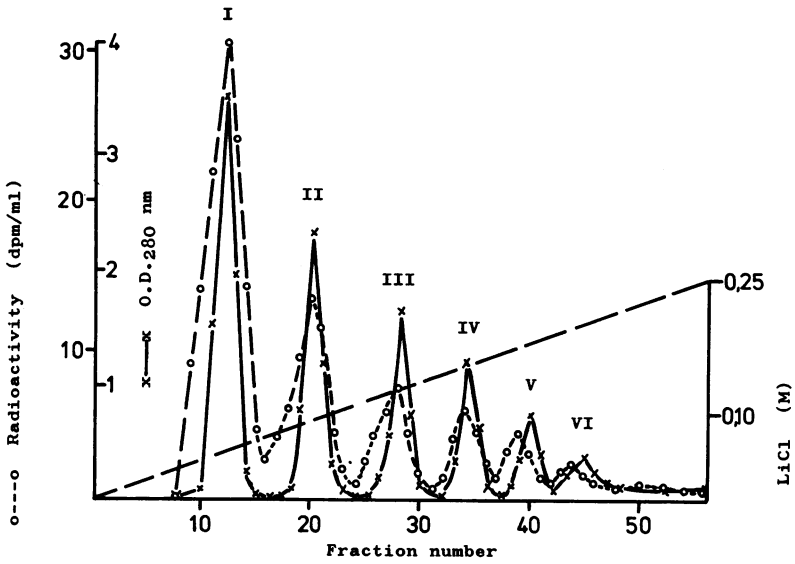

Fig. 5: Profile of the pyrimidine isostichs of M. luteus DNA methylated by rat liver methylase.
0.5 µg denatured M. luteus DNA was methylated as described for fig. 2 and, after the addition of 2 mg salmon sperm DNA, the pyrimidine isostichs were prepared and separated on a 2 ml DEAE-cellulose column in a 2 ml pipette as described in methods. The total volume of the gradient (0 - 0.25 M LiCl in 0.025 M Na acetate, pH 5.2) was 100 ml. 1.5 ml fractions were collected.

as in vivo. In our opinion this is in favour of only one methylase with low sequence specificity or at least a rather limited number of enzymes. Our first attempts to determine the dinucleotides methylated by the enzyme at different purification stages and an isostich analysis for the purified methylase from Krebs ascites cells (26) support such an interpretation.

The comparison of different substrates (tab. II) showed that the natural DNAs were all better acceptors of methyl groups in ss than in the ds form.

But as verified with S_1-nuclease, ds unmethylated M. luteus DNA as well as ds unmethylated ascaris suis DNA could be methylated at a considerable rate and to a high extent. These results argue against a role of the enzyme in the differentiation model of Holliday and Riggs (5,6).

Eukaryotic DNA methylases described so far have been shown to contain CpG in their recognition sequence (9,28,29). But whether all the CpGs are methylated or whether further sequence

TABLE II. Substrate specificity of rat liver DNA methylase.

Substrate	unheated		heated	
	cpm	% of M. luteus	cpm	% of M. luteus
M. luteus DNA	6164	100	24352	100
E. coli B DNA	2153	35	10870	45
Ascaris suis DNA	3820	62	-	-
Rat liver DNA	170	2.8	410	1.7
PM2 DNA	2850	46	-	-
SV 40 DNA	93	1.5	-	-
(dG-dC)n · (dC-dG)n	9800	160	2140	9
(dG)n · (dC)n	0	0	-	-
(dC)n	-	-	0	0
(dG,dC)n	11500	187	5020	21
(dA-dC)n · (dT-dG)n	0	0	0	0
(dC,dT)n	580	9.4	0	0

5 µg of substrate was incubated with the purified enzyme under standard assay conditions for ss and ds substrate respectively, as described in methods.

requirements are necessary is not yet clear.

The DNA methylase from rat liver was able to methylate the copolymer (dG-dC)n•(dC-dG)n, which speaks in favour of a recognition sequence composed of alternating G and C.

The methylation extent of q in 17 bases of M. luteus DNA indicates a recognition sequence shorter than a trinucleotide. Although the methylation extent is at least 7-fold higher than reported for other DNA methylases from eukaryotic cells, we assume that the DNA is not yet completely methylated. As preferential methylation of the 3'end of isopyrimidine stichs has been documented (25), the isostich analysis demonstrates, besides methylation of the sequence Pu CpG (monopyrimidine stich), a considerable degree of methylation in CpCpG or TpCpG or both. An alteration of the sequence specificity in vitro can be excluded by the fact that homologous rat liver DNA accepted only 1 methyl group in about 600 bases.

From these considerations we propose that the main re-

cognition sequence of the DNA methylase from rat liver and probably of other eukaryotic DNA methylases as well is CpG. Methylation of all the CpGs agree well with the amount of CpG in eukaryotic DNA (about 1 %) (30) compared to the amount of methyl groups (0.85 % for rat liver DNA). It is further supported by a restriction enzyme analysis of calf thymus satellite DNA (31). On the other hand the finding of methyl groups in the CpT dinucleotide and in internal C of pyrimidine isostichs in Novikoff cells (25) does not fit into the above concept and needs further clearification.

As the methylase from Krebs II cells (11) the rat liver methylase, too, failed to methylate SV40 DNA to a significant degree, but efficiently methylated PM2 phage DNA even in the supercoiled form.

As the small genome of mammalian viruses contain CpG, although in a reduced amount, we assume that, besides the methylation site CpG, the methylase needs a more complex sequence to bind specifically to DNA. Once specifically bound to DNA the methylase would be able to move along the DNA and methylate CpG, as proposed by Drahovsky and Morris (12) and supported by our results (fig. 3). Concluded from the methylation of (dG-dC)n •(dC-dGn) the binding sequence should be composed of C and G. In the small viruses of eukaryotes there could have been a selection against similar sequences.

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Dedicated to Professor Dr. Dr. med. h.c. Otto Westphal on the occasion of his 65th birthday.

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